REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 2003	Annual (1 Sep 2002 - 31 Aug 2003)

4. TITLE AND SUBTITLE

Role of Casein Kinase 1 and DARPP-32 in Parkinson's Disease

5. FUNDING NUMBERS

DAMD17-02-1-0705

6. AUTHOR(S)

Paul Greengard, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Michael Stern Parkinson's Research Foundation
New York, NY 10036

E-Mail: greengd@mail.rockefeller.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

Original contains color plates: ALL DTIC reproductions will be in black and white

12a. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

One of the goals of Parkinson's disease (PD) research is to delay and/or moderate the symptoms of PD that result from the effects of dopamine loss. During the past year, scientists at the Stern Foundation have identified a key molecular target that can potentially allow dopaminergic neurons to provide adequate dopamine signaling in the presence of decreased dopamine availability. Specifically, it was previously known that DARPP-32 potentiates dopamine signaling by inhibiting the phosphatase, PP-1. This is determined by phosphorylation of DARPP-32 at Threonine-34. However, the PP-1 inhibitory activity of Thr-34 phosphorylated DARPP-32 is limited by the phosphatase, PP2B, which dephosphorylates DARPP-32 at Thr-34. We have shown that phosphorylation of DARPP-32 at Ser-130 by casein kinase 1 (CK1), inhibits dephosphorylation of Thr-34 by PP-1. This effect can be mediated by amphetamine, which causes phosphorylation of DARPP-32 at Ser-130.. We used transgenic DARPP-32 knockout mice, DARPP-32 Ser/ALA-34 mice (the mutation inactivates the Ser-130 site) and WT mice to elucidate the effect. The implication of this work is that inhibitors of PP2B or other means by which DARPP-32 phosphorylation at Ser-130 can be stimulated could allow DARPP-32 to compensate for loss in dopamine production and thus ameliorate PD symptoms.

14. SUBJECT TERMS

Parkinson's Disease

15. NUMBER OF PAGES

10

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

AD_____

Award Number: DAMD17-02-1-0705

TITLE: Role of Casein Kinase 1 and DARPP-32 in Parkinson's Disease

PRINCIPAL INVESTIGATOR: Paul Greengard, Ph.D.

CONTRACTING ORGANIZATION: Michael Stern Parkinson's Research Foundation
New York, NY 10036

REPORT DATE: September 2003

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

Table of Contents

Michael Stern Parkinson's Research Foundation
Progress Report for Period 9/1/02 – 8/31/03
Contract # DAMD17-02-1-0705
Principal Investigator – Paul Greengard

Involvement of CK-1/Ser-130-DARPP-32 in the biochemical and behavioural effects of the dopamine-releasing agent, amphetamine.

INTRODUCTION

Parkinson's disease affects 1% of the population above the age of 65. The major neurological impairments are intimately related to the progressive degeneration of dopaminergic neurons. In order to develop improved treatments for Parkinson's disease, it is crucial to increase our fundamental understanding of dopaminergic neurotransmission.

By using mice with targeted deletion of the gene encoding DARPP-32, we have demonstrated that DARPP-32 plays a key role in the actions of dopamine. The present proposal is devoted to studies of the effects of dopamine and other neurotransmitters acting via DARPP-32 in CK-1 mutant mice and animal models of Parkinson's disease.

BODY/KEY RESEARCH ACCOMPLISHMENTS

Research objectives 1
Generation of mice overexpressing CK-1

Research objectives 1 and 2 require the construction of an elaborate set of mouse models that have each isoform of CK-1 (casein kinase 1) knocked out by homologous recombination and another set of mice that overexpress each isoform. Below we discuss the progress made on the overexpressor mice in Section A and then follow with the progress on the knockout of all four isoforms of CK-1 which include the delta, epsilon, alpha and gamma-1 isoforms.

A. CK-1 inducible overexpression

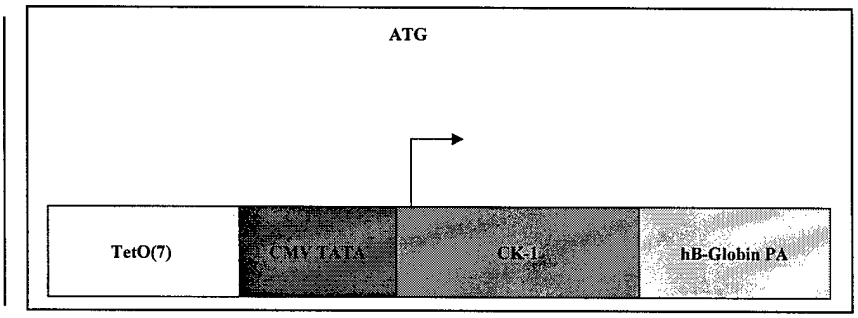
cDNA isolation: Mouse brain mRNA was bought from Clontech and cDNA was prepared. Forward and reverse primers matching published sequences were used in PCR reactions to amplify CK1- delta, CK-1 epsilon, CK-1 alpha and CK-1 gamma coding regions. The cDNA's were subcloned and sequenced to ensure no errors were introduced by PCR.

For CK-1 delta: cDNA coding region was isolated matching gene accession no NM_139059 describing isoform CK-1 delta 1 (csnk1δ1)
For CK-1 epsilon: cDNA coding region was isolated matching gene accession no NM_013767 describing isoform CK-1 epsilon (csnk1ε)
For CK-1 alpha: cDNA coding region was isolated matching gene accession no NM_146087 describing isoform CK-1 alpha 1 (csnk1α1)
For CK-1 gamma: cDNA coding region was isolated matching gene accession no NM_173185 describing isoform CK-1 gamma 1 (csnk1γ1)

Transgene construction: The coding region of each isoform was cloned into a tetracycline or doxycycline-inducible expression vector (pTRE2, Clontech catalog no 6241-1) to create pTRE-2 csnk1δ1, pTRE-2 csnk1ε, pTRE-2 csnk1α1, pTRE-2 csnk1γ1, **Fig. 1**. The pTRE-2 expression vector has a minimal CMV promoter preceded with seven copies of the 42pb tet operator sequence (tet0). At its 3'end it harbors the human Beta globin polyA.

Expression vectors pTRE-2 csnk1δ1, pTRE-2 csnk1ε, pTRE-2 csnk1α1 and pTRE-2 csnk1γ1 were purified, vector sequences removed and mouse pronuclei were injected. For TRE-2 csnk1δ1, one animal has been identified carrying the expression vector (July, 03), with an estimated copy number between one and ten. Presently we are in the process of genotyping 17 pups born from TRE-2 csnk1δ1 pronuclei injections, and 4 pups born from TRE-2 csnk1e pronuclei injections. TRE-2 csnk1α1 and TRE-2 csnk1γ1 have been injected, and we are waiting for births.

Fig 1. pTRE-2 CK-1 transgenes



CK-1 targeting

Genomic cloning: The genomic region of csnk1δ1, csnk1ε, csnk1α1 and csnk1γ1 has been isolated using either BAC library screens and/or published sequences. The resulting genomic structure of the four CK1 isoforms is described in **Fig.2.**

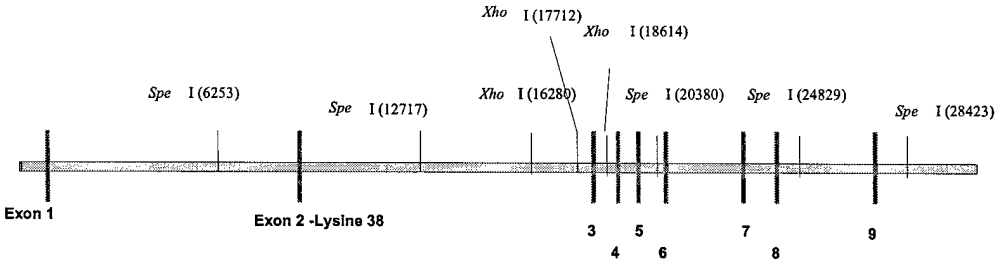
Construction of targeting vectors: One targeting vector (TV) per isoform was created using homologies with as long a sequence as possible (TVcsnk1δ1, TVcsnk1ε, TVcsnk1α1, TVcsnk1γ1). A neomycin Resistance (neo^R) selection cassette flanked with two FRT sites and one lox site at its 3' end was inserted into intron regions downstream of the ATP binding domain and one 5' lox site was inserted upstream of the ATP binding domain (**Fig 3**). This will leave two possibilities: 1. upon CRE enzyme application in ES cells, and following site-specific recombination a deletion will be created, leading to a possible null phenotype in mice. 2. Upon Flp enzyme application in ES cells and following site-specific recombination, the neo^R selection cassette will be excised leaving just two lox sites. Mice carrying two lox sites will be used for Cre recombination in vivo by mating them with mice carrying Cre.

TVcsnk1γ1, TVcsnk1ε have been electroporated, selected and neo^R ES colonies have been isolated. ES colonies have been cryopreserved and duplicate colonies are presently grown for genomic DNA isolation. TVcsnk1δ1 and TVcsnk1α1 are scheduled for electroporation in the next month. Given the complexity of the casein kinase gene family good progress has been made on this project and we foresee no complications.

Fig 2. Genomic structure of Cskn1δ1, Cskn1ε, Cskn1α1, Cskn1γ1,

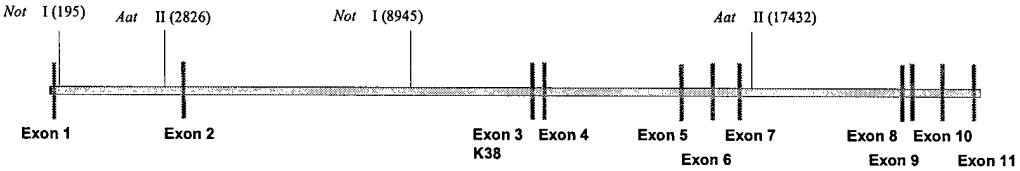
Csnk1 Delta

Genomic organization30kb



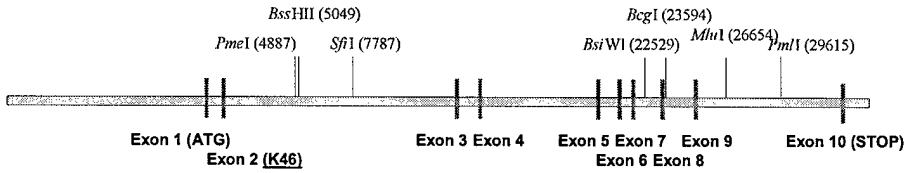
Csnk1 Epsilon

Genomic organization 23,100 bp



Cskn1 alpha

Genomic organization 33 kb



Csnk 1 gamma 1

Exon 3,4,5 ~22.6 kb
whole genomic organization of Csnk1 gamma: 14 Exons in 132 kb

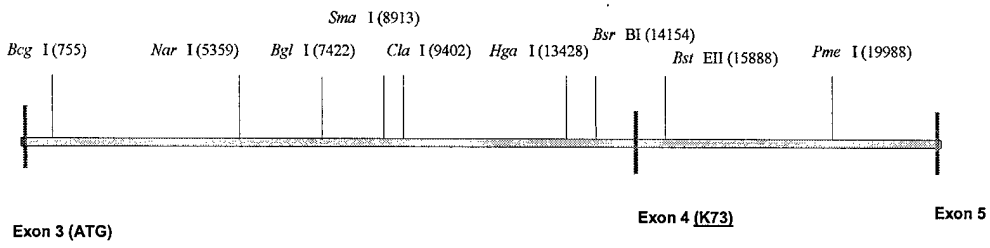
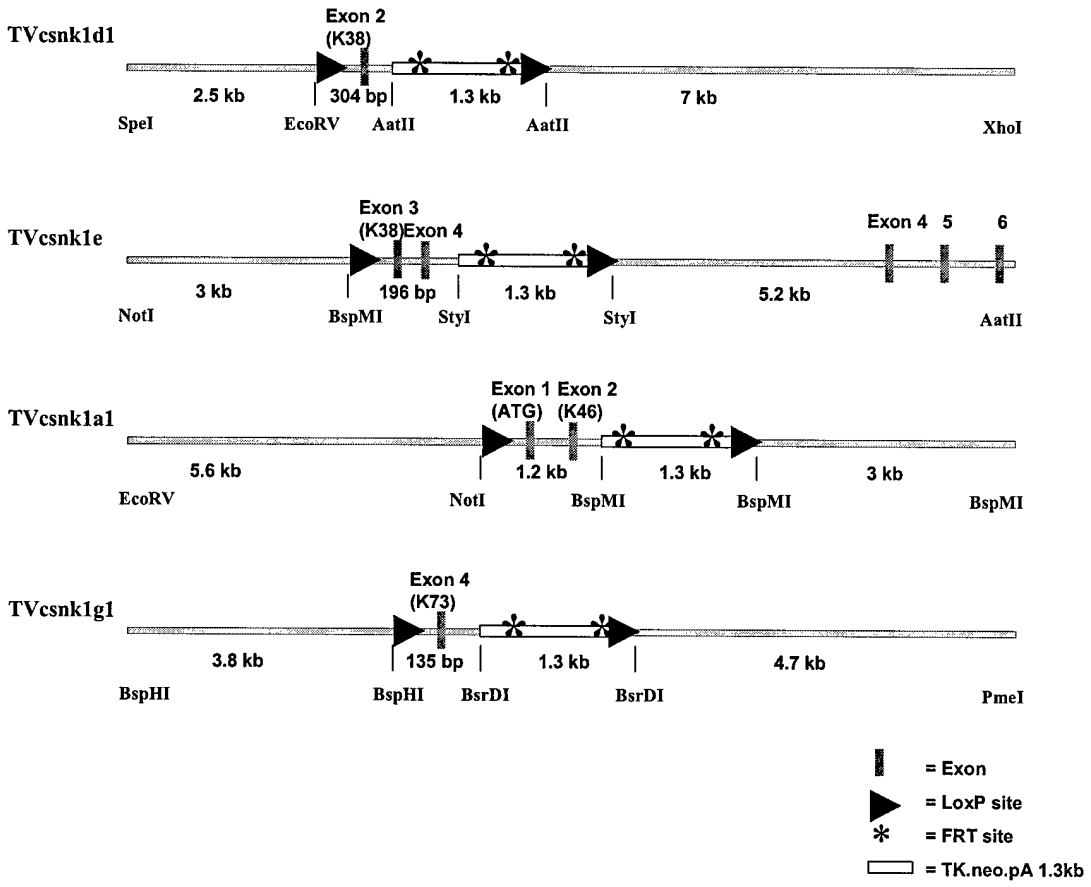


Fig 3. Targeting vectors



Research objective 2
Studies of L-DOPA and of dopamine receptor ligands in traditional animal models of PD

During this first year, in addition to using traditional animal models of PD, we have studied animal models with specific CK-1 target site modifications. The role of CK-1 in dopaminergic neurotransmission is largely unknown but DARPP-32, a key protein that potentiates the effects of dopamine, is known to be phosphorylated by CK-1 at position Ser130. Therefore, wild-type mice and DARPP-32 knockout (KO) mice were used together with "knockin mice" in which a single point mutation (Ser130Ala-DARPP-32) was engineered. In wild-type mice, we found that amphetamine increased the level of phosphorylated Ser130-DARPP-32 in several brain regions, including frontal cortex, striatum and cerebellum. In order to examine the functional importance of the increased Ser130-DARPP-32 in the action of amphetamine, we studied the effects of amphetamine on movement stimulation in normal wildtype (WT) mice, in DARPP-32 knockout (KO) mice and in knockin mice in which Ser130 has been replaced with an alanine (S130A mutants). It was found that amphetamine stimulated movements in WT mice. However, this stimulatory effect of amphetamine was greatly diminished in DARPP-32 KO mice and, interestingly, also in S130A mutants.

Thus, the development of the novel gene knock-in methodology used in the present study to engineer mice with a point mutation at Ser130 in the DARPP-32 molecule has led to the unexpected finding that Ser130-DARPP-32 plays a major role in the actions of dopaminergic neurotransmission. As described above, based on in vitro studies, we have reported that CK-1

prevents the dephosphorylation by PP-2B of P-Thr34-DARPP-32. The S130A mutant mice have allowed us to evaluate directly whether Ser130-DARPP-32 primes phosphorylation at Thr34-DARPP-32. For this purpose, we studied the phosphorylation at Thr34-DARPP-32 in S130A-mutants under basal conditions and after administration of amphetamine. It was found that amphetamine induces significantly more Thr34-DARPP-32 in controls than in S130A-mutants in striatum, supporting a modulatory role for Ser130-DARPP-32 in the regulation of Thr34-DARPP-32 phosphorylation. The data indicate a hierarchical interdependence between Ser130- and Thr34-DARPP-32 and support an important role for CK-1 in the actions of amphetamine.

We are currently examining the involvement of Ser130-DARPP-32 in the actions of other agents that stimulate dopamine neurotransmission in normal animals and in animal models of Parkinson's disease.

Research objective 3
Studies of compounds acting via receptors for adenosine, serotonin, glutamate and opioids in traditional animal models of PD

As a part of our effort to understand serotonergic signaling, we have previously examined the action of serotonin on phosphorylation of DARPP-32 at Thr34, Thr75 and Ser130 in striatal slices made from MPTP pretreated mice (Svenningsson et al., 2002). We have now extended this analysis to studying effects in the 6-OHDA lesioning model of Parkinson's disease. We have compared the effect of serotonin on phosphorylation of DARPP-32 at Thr34, Thr75 and Ser130 in the intact and dopamine-depleted hemispheres. Results from both these models indicate that serotonin regulates DARPP-32 phosphorylation in a similar manner under normal conditions and in animal models of Parkinson's disease. Moreover these results indicate that serotonin regulates DARPP-32 phosphorylation independent from dopamine. Serotonergic compounds could therefore be useful as adjuvant treatment for Parkinson's disease. Further studies with selective pharmacological tools are currently being performed.

Research objective 4
Generation of constructs and yeast two-hybrid screens for CK-1

During this year, we have cloned from different libraries the full length CK1 cDNAs corresponding to several isoforms (α, γ, δ and ε). We have made the bait constructs by ligating those cDNAs downstream and in-frame with the GAL4 DNA binding domain in the yeast expression vector pAS2. The constructs have been sequenced and characterized in terms of toxicity and auto-activating activity in yeast after transformation into the *S. cerevisiae* reporter strain CG1945 (Clontech). The level of expression and the size of fusion proteins have been confirmed by Western blotting with antibodies directed against the GAL4 DNA binding domain. All the baits are correctly expressed and the expression levels are compatible with screening requirements.

Screens have been done using yeast two hybrid rat cDNA libraries and using the Gal4 based system. All the baits have been used at least once for screening the libraries. For each bait, we screened between 25 and 150 million diploid cells (at least 8 times the complexity of the library). The background of these libraries (mainly due to false positives interacting with GAL4 domain and "sticky" candidates) is well characterized in our laboratory. The clones corresponding to this type of candidates have not been considered for further characterization. At this level of analysis, the CK1-α and CK1-γ screens have not been studied further because of the small number and the nature of the candidates obtained. For CK1-δ and CK1-ε, the remaining preys have been

classified into categories of distinct heuristic values considering the number of identical or independent clones found for the same candidate, the frame of the fusion protein, the size of the insert, and the relevance of the putative candidates. Taking this classification into account, interesting preys have been reconfirmed in yeast and tested for specificity against different irrelevant baits, including the original empty bait plasmid. Candidates found that correspond to published interactions have not been kept for further characterization. For example, the Axin protein, which is known to interact with and to be phosphorylated by CK1, has been found several times (17% of the clones found for CK1-δ). Per1, a protein regulating the circadian clock and a substrate of CK1-δ has been found with the CK1-δ bait. These previously identified CK1 interacting proteins confirm the validity and the potency of our method.

For the **CK1-δ screen**, 5 strong candidates are remaining:
- Four of them correspond to unknown proteins. They represent 60% of the 102 clones analyzed and we found at least 8 independent clones for each of those candidates.
- The last one is a new protein, known as a potent tumor suppressor and connected to the Ras-Raf signal transduction pathway; this candidate represents 17.6 % of the clones.

For the **CK1-ε screen**, one major candidate has been found. This candidate represents 65% of the 126 clones analyzed and is represented by many different 5' junction giving this candidate a very strong heuristic value. It is an unknown protein harboring a strong cyclin signature. Besides this candidate, 4 other minor ones have been found (less than 5 times each).
In summary, we have finished the analysis of the different screens and we have started to further characterize several candidates. We are now verifying their specificity and cloning the full length cyclin domain containing candidate. This candidate, because of the presence of the cyclin domain, could potentially be a CK1 regulator. This is particularly interesting as the mechanism of regulation pf CK1 activity remains unknown.

REPORTABLE OUTCOMES

No publications have resulted from these studies to date.

CONCLUSIONS

During this first year we have cloned all CK-1 isoforms from mouse and rat cDNA libraries. We have made substantial progress in the generation of several CK-1 mutant mice. We have found evidence of the importantance of the role the DARPP-32 CK-1 phosphorylation site (Ser130) plays in dopaminergic transmission. We have also shown serotonin regulates DARPP-32 phosphorylation in a similar manner under normal conditions and in animal models of Parkinson's disease. Finally we have started several yeast two hybrid screens with different CK-1 isoforms. We have found interesting candidates, one of them having a cyclin homology, which is a possible CK-1 regulator.

FUTURE EXPERIMENTS

During the next budget period construction of the vectors needed for the different mutant mice will be completed. We will genotype the pups born for the CK-1 inducible overexpressing mice project. New births are expected in the next few months. Colonies for TVcsnk1g1 and

TVcsnk1e will be further characterized and we should be able to electroporate the constructs for the two remaining CK-1 isoforms constructs (TVcsnk1a1 and TVcsnk1d1).

Studies of dopamine receptor ligands in animal models of PD as well as ligands acting via other receptors (serotonin, glutamate, adenosine and opiods) will continue. Biochemical analysis of the first mutant mice available should begin midway through the year depending on preliminary characterizations and mouse breeding efficiency.

Studies of traditional animal models of PD will continue using serotonin and studies using compounds acting via the receptors for adenosine, glutamate and opioids will commence.

As soon as we have the full length cDNA for the cyclin-like candidate, 1) we will express it in *E. coli* as a GST-fusion protein to confirm the interaction in a different system (GST pull-down), 2) we will use it as an antigen to prepare rabbit polyclonal antibodies, 3) we will investigate whether the interaction of CK1-ε with the cyclin-like candidate can be detected in mammalian tissue by immunoprecipitation, 4) we will study the influence of the cyclin-like protein on the catalytic activity of recombinant and native CK1, and 5) we will investigate the tissue and cellular distribution of the cyclin-like protein. In a parallel manner, we will confirm the CK1-δ interaction with the candidate proteins and check their specificity in yeast. For all the candidates we will analyze the tissue distribution by Northern blot, and the brain distribution by immunohistochemistry with the antibodies that we will make, focusing our attention specifically on the striatum.

REFERENCES

Per Svenningsson, Eleni T. Tzavara, Feng Liu, Allen A. Fienberg, George G. Nomikos, and Paul Greengard, DARPP-32 mediates serotonergic neurotransmission in the forebrain, PNAS 2002; 99: 3188-3193.